



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Metal internalisation by bacterial cells depends on metal biotoxicity and metal to biomass ratio.

Citation for published version:

Liang, L & Ngwenya, B 2018, 'Metal internalisation by bacterial cells depends on metal biotoxicity and metal to biomass ratio.', *Chemosphere*. <https://doi.org/10.1016/j.chemosphere.2018.08.125>

Digital Object Identifier (DOI):

[10.1016/j.chemosphere.2018.08.125](https://doi.org/10.1016/j.chemosphere.2018.08.125)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Chemosphere

Publisher Rights Statement:

© 2018 Elsevier Ltd. All rights reserved.

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



1

2 **Title:** Metal internalisation by bacterial cells depends on metal biotoxicity
3 and metal to biomass ratio.

4

5 **Author:** Lili Liang¹, Bryne T. Ngwenya²

6

7 **Author address:**

8 ¹School of Environmental Studies, University of Geoscience, Wuhan,
9 430074, China.

10 ²School of Geosciences, University of Edinburgh, James Hutton Road,
11 Edinburgh EH9 3FE, United Kindom.

12

13 **Corresponding author:** *Lili Liang and Bryne T. Ngwenya*

14 **E-mail:**

15 **Lili Liang:** lianglily99@126.com

16 **Bryne T. Ngwenya:** Bryne.Ngwenya@ed.ac.uk

17

18

19

Metal internalisation by bacterial cells depends on metal

biotoxicity and metal to biomass ratio

Lili Liang^{1} and Bryne T. Ngwenya^{2*}*

¹School of Environmental Studies, University of Geoscience, Wuhan, 430074, China.

²School of Geosciences, University of Edinburgh, James Hutton Road, Edinburgh EH9 3FE, United Kingdom.

* Corresponding author.

Abstract

The traditional view of metal adsorption to bacterial surfaces is that it can act as a protective mechanism by externalizing the metal outside the cell. However, numerous studies focussing on the biodynamics of metal uptake using biotic ligand models consider metal adsorption to cell surfaces as an important first step in metal uptake and internalisation. In order to resolve these conflicting views, we adsorbed two metals (copper and cadmium) with contrasting metal biotoxicity on *E. coli JM109*, and quantified the distribution of each metal amongst surface sites, periplasmic space and the cytoplasm. Distribution of each metal depended on biotoxicity and metal to biomass ratio. For both metals, low metal to biomass ratio led to most of the metal being associated with the periplasmic space, with less Cd being taken up by cells overall. At high metal to biomass ratios, most of the Cd was associated with surface sites, whereas

Cu also increased in surface sites but remained below periplasmic concentrations. These observations are consistent with metal internalization being the dominant process at low metal to biomass ratios, whereas was active efflux when metal to biomass was high, leading to equilibrium between cytoplasm and surface concentrations. Significantly, efflux was more intense for high biotoxicity Cd, consistent with active enzymatic regulation of Cu internalization/homeostasis, which is essential at low concentrations. Moreover, metal internalization increases as surface-bound metal increases, the maximum being constrained by maximum adsorption consistent with Langmuir adsorption behaviour.

Summarize of paper: Bacterial metal internalisation is a function of metal biotoxicity and metal loading.

Key words: Metal internalisation; Bacteria; Adsorption; Uptake; *E.coli JM109*

1. Introduction

Amongst many geomicrobiologists, the traditional view of metal interaction with bacterial cells is that the metal associates with the cell by biosorption, involving non-specific binding to cell surfaces (Gadd, 2007; Fomina & Gadd, 2014). This has led to a proliferation of studies employing surface complexation models as a basis for

developing predictive approaches to quantifying metal uptake, with little to no regard for cellular internalisation of the metal (Fein et al., 1997 & 2001; Ngwenya et al., 2003; Borrok et al., 2005; Tourney et al., 2009; Ngwenya et al., 2010; Kenney and Fein, 2011). By integrating biosorption studies with spectroscopic measurements (e.g. infra-red and X-ray absorption spectroscopy), these studies have identified specific functional groups that are responsible for metal complexation at the cell surface, including carboxyl, phosphate, amine, phosphodiester and sulfhydryl moieties.

In contrast, there have been numerous and independent studies by biogeochemists focussing on the biodynamics of metal uptake by micro-organisms (including bacteria and algae), in which biosorption is considered only as a first step in metal bioaccumulation, ultimately leading to metal internalisation by cells (Campbell et al., 2002; Duval et al., 2014, 2015, 2016; Rotureau et al., 2015; Present et al., 2017). Building on such approaches, the study by Pabst et al (2010) was one of the first to attempt to quantify metal partitioning between different cellular compartments in a bacterial cell, using selective extraction to demonstrate that cadmium and copper associated differently with the cell surface, periplasm and cytoplasm of the bacteria tested. Specifically, cadmium was mainly found on the surface of the cell, ascribed to an active metal efflux mechanism due to cadmium's toxicity profile. It was thought that the cadmium was bound to the surface functional groups identified from surface complexation models. Such studies provided the platform for recent attempts to combine surface complexation, initial bulk metal concentration and biotic ligand

models (BLM), providing a means to predict metal toxicity purely from surface complexation models (Flynn et al., 2014; Duval et al., 2015; Fein, 2017).

The aim of this study was to probe deeper into the mechanism behind differences in metal compartmentalisation linked to the biotoxicity profile of the metal. We used the same metals (copper and cadmium) as Pabst et al (2010) but on a different bacterium, *E. coli* JM109. We specifically sought to vary metal to biomass ratio, hypothesising that active metal efflux processes would only be triggered above certain concentration thresholds with little or no control from surface chemistry, as suggested by Pabst et al (2010).

2. Materials and methods

2.1 Cell growth and preservations:

Experimental batches of *E.coli* JM109 were grown from the primary culture (stocks) by first plating them overnight in nutrient agar, transferring and growing a single colony overnight in 100ml nutrient broth No.3, and using the overnight culture to inoculate 2L Pyrex flasks containing 1 litre of nutrient broth No.3. Cultures were incubated on a shaker for 48 h at 30 °C. Cells were harvested by centrifugation at 11000g for 10 min at 4 °C. The harvested cells were then washed three times in 18 MΩ ultrapure water, freeze-dried and homogenised into powder for use in the experiments described below. Previous studies have shown that most of the cells prepared and preserved by this method are intact and viable (Ngwenya et al., 2003).

2.2 Metal exposure solutions

The metal solutions were prepared in 20mL Teflon centrifuge tubes at concentrations ranging from 0.5 to 10 mg/L (0.008 to 0.16mM) of free Cu^{2+} ions using $\text{Cu}(\text{NO}_3)_2$, and Cd from 0.5 to 10mg/L (0.004 to 0.1mM) of Cd^{2+} using $\text{Cd}(\text{NO}_3)_2$ in a inert background electrolyte of 0.01M NaNO_3 , chosen to limit solution complexation of the metals. This means that although cells were viable (Ngwenya et al., 2003), they were not metabolising. This need not necessarily curtail active enzymatic responses, as shown by previous studies where inert exposure media have been used (e.g. Smeijian et al., 2000). The pH in each tube was adjusted to 5, and geochemical modelling using Visual Minteq showed that both metals were in free ionic form at this pH, and that over 90% each metal is adsorbed to cells at pH 5 (Parker et al., 1995; Ngwenya et al., 2010).

2.3 Metal adsorption experiments

Adsorption experiments were conducted at two different cell (biomass) suspension concentrations, 0.5g/L and 2g/L, in order to compare the effect of different metal to biomass ratios, by suspending 10mg and 40mg of freeze-dried bacteria in 20mL of the metal solution. Experiments were performed in triplicate. Tubes were mixed on a carousel at $25 \pm 1^\circ\text{C}$ for 30min and then centrifuged to pellet the cells and obtain a supernatant. Previous studies (Wu et al., 2009; Langley and Beveridge, 1999; pabst et al., 2010) and in our laboratory (Ngwenya et al., 2003) have shown that this contact time is sufficient for adsorption to reach equilibrium. Chemical buffering agents were not added during the whole procedure, and pH was maintained throughout the study to

within 0.2pH units. Following equilibration, cells were separated from the supernatant by centrifugation at 11000g for 10 min at 4 °C. The cell pellet was used for compartmentalisation experiments described below. The supernatant was filtered, acidified to 2% v/v with nitric acid, and analyzed for Cu or Cd using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES, PerkinElmer 5300DV). Analytical quality on the ICP-OES was checked against a certified mixed metal standard (M6, Merck), yielding precision better than 2% relative standard deviation and accuracy to within 1% of the certified values.

2.4 Characterization of metal-cell compartmentalization

We adopted the method of Pabst et al (2010) and Mclean et al (2013), but with slight modifications, to determine metal-cell compartmentalization following adsorption by measuring the proportion of Cu and Cd associated with surface exchange sites (q_{exch}), the periplasmic space (q_{peri}) and the cytoplasm (q_{cyt}). Metal associated with exchange sites on the cell surface were extracted by suspending the cell pellet into 20mL of 5mM Ca^{2+} as $\text{Ca}(\text{NO}_3)_2$ which had been adjusted to pH 5 on a carousel at $25 \pm 1^\circ\text{C}$ for 20min (salt et al., 1997). The suspension was centrifuged at 11000g at 4°C and the supernatant analyzed for the desorbed metal. The step for determining outer membrane and periplasmic metal compartmentalization was modified by increasing the extraction time in 20ml of 20 mM EDTA (adjusted to pH 5) from 20s (Pabst et al., 2010) to 20 minutes to allow longer equilibration. This is because most bacterial cells contain a certain amount of EPS, and since EDTA is one of the reagents used to disrupt EPS

(Liu et al. 2002; Tourney et al., 2008; D'Abzac et al., 2010), we assumed that this step should also extract metal bound to EPS but not exchanged with Ca in the first step, allowing for the fact that metal movement/extraction from EPS can be affected by slow diffusion (Ha et al., 2010). After centrifugation, the remaining pellet was digested with 3mL concentrated nitric acid, heated to 120°C for 5min, then dried and re-dissolved in 20 ml of 2% HNO₃ for ICP-OES analysis. The sum of Cu and Cd from the three extraction steps was designated as the total amount of metal associated with the cell, q_{frac} . Data quality was checked by mass balance calculations of the three compartments, which was between 90% and 110% when the initial adsorbed concentration and the sum of extracted metals were compared.

2.5 Acid-base titrations and surface complexation modelling

Potentiometric titrations were performed on suspensions of each bacteria following the protocol of Ngwenya et al (2003) in order to characterize the number and types of proton-active functional groups present on cell surfaces. Briefly, titrations were carried out at room temperature on 50-ml suspensions of the bacteria in a background electrolyte of 0.01M NaNO₃, which had been purged of dissolved gases with O₂- and CO₂-free N₂ for 30 minutes. Titrations were conducted using an automated DL53 (Mettler Toledo) burette assembly using 0.1M NaOH following acidification of the suspension to pH ~3. The titrant was standardised in triplicate against potassium hydrogen phthalate (KHP) and the mean concentration of the three standards used in the calculations. The titrations were carried out in polypropylene beakers sealed to

atmospheric gases and a positive N₂ pressure was maintained throughout the titration. The titrator was programmed in dynamic mode and successive titrant additions were made only when a stability of 0.1mV/s had been attained. Base consumption data in the form of H⁺ adsorbed was normalised to the dry mass concentration of bacteria (10g/L) and modelled using FITEQL4 (Herbelin & Westall, 1999) to calculate deprotonation constants and surface densities of proton-active sites.

2.6 Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared (FTIR) spectroscopy was performed on cells before and after adsorption of 5mg/L of each metal in order to detect functional groups that may contribute to metal binding/adsorption by cells to complement potentiometric titrations. To prepare samples, 0.01g of freeze-dried cells were mixed with 20 ml solutions of 5mg L⁻¹ of Cu(NO₃)₂ and Cd(NO₃)₂ in 0.01M NaNO₃ background electrolyte and adjusted to pH 5. Samples were performed in triplicate, and tubes were mixed on a carousel at 25±1°C for 30min, then centrifuged to pellet and the pellet freeze-dried. The prepared samples, together with controls (freeze-dried cells without metal adsorption) were mixed with KBr in the ratio of 1:100 and pellets of 13mm diameter were prepared at 8×10³ kgcm² pressure. The IR spectra were carried out on a Nicolet 6700 (Thermo Scientific, USA) spectrometer over the wave number range of 400-4000 cm⁻¹. For each sample, 40 scans were collected at a resolution of 0.09 cm⁻¹ and spectra added.

2.7 Data analysis.

The amount of metal adsorbed (q_{ads} , mmol/kg) for each initial metal concentration was calculated by difference between initial and equilibrium metal concentration (C_e , mmol/L). Since pH was maintained constant within experimental limits, data was analyzed in the form of pH isotherms where bulk adsorbed (q_{ads}) and compartmentalised (q_{exch} , q_{peri} , and q_{cyt}) metal contents were plotted against equilibrium metal concentrations in solution (Figure 1). In this form, adsorption displayed linear behaviour when metals were equilibrated with 2g/L cell suspensions, leading to pseudo-linear distribution coefficients (Figure 1A and 1B)). For 0.5g/l cell suspensions, the relationships were non-linear and hence a one site Langmuir model was used to compare parameters for the distribution of metal adsorption to cells (Figure 1C and 1D). The Langmuir model is given in equation 1 and relates the amount of metal adsorbed (q) in mmol·kg⁻¹ and the concentration of the metal in solution at equilibrium:

$$q = \frac{Q^0 b C_{eq}}{1 + b C_{eq}}$$

where Q^0 is the maximum mass adsorbed at saturation conditions per mass unit of adsorbent (mmol·kg⁻¹), and b is an empirical constant with units of inverse concentration (Bubba et., 2003; Mclean et al., 2013).

3. Results and discussion

3.1 Metal adsorption and compartmentalization.

Bulk adsorption isotherms for the two metals and at different metal to biomass ratios were presented Figure 1, which also plots the sum of fractionated metal (q_{frac}) against bulk equilibrium concentrations and associated fitted parameter were presented Table 1 and 2. Whilst this is not evidence that the extraction steps are uniquely selective to the specific compartments, the data clearly shows that the extractions are quantitative and robust, being within experimental/analytical error.

For the fractionated data, we noted differences in the behavior of copper and cadmium, as well as in the metal uptake behavior that depended on the amount of cells equilibrated with the same initial metal solution concentrations. Consequently, we describe copper and cadmium results separately, by comparing/contrasting cell to biomass ratio, with lower concentrations defined as those with low metal to biomass ratio.

3.1.1 Copper.

At low metal to bacteria ratios (2g/L cells), copper isotherms are nearly linear (Figure 2A,). Most of the Cu resides in the EDTA-extractable fraction, considered to represent Cu bound to periplasmic space and the concentration in this fraction increases steeply with external (equilibrium) copper concentration. Surface adsorption (q_{exch}) represents the lowest fraction in both types of cells and this fraction is only weakly dependent on external copper concentrations.

At high metal to biomass ratios (0.5g/L bacteria), the adsorption isotherms are non-linear and all individual compartments display Langmuir behaviour (Figure 2C,).

consistent with a limiting uptake value as concentrations in solution increase. However, the highest concentrations still occur in the EDTA-extractable fraction (periplasmic space), particularly at low equilibrium concentrations (Table 1). In addition, the exchangeable fraction is now a significant proportion of the total Cu uptake whereas the cytoplasmic fraction remains fairly low, overtaking the periplasmic fraction only at high equilibrium concentrations, although data at high equilibrium concentrations is limited. Such an observation is similar to that of Pabst et al (2010) who reported that for the KT2440 pseudomonad, more copper was associated with the periplasmic space at low equilibrium solution concentrations whereas the proportion of Cu on the surface increased relative to that in the periplasm with increasing solution concentration.

Thermodynamic models of metal uptake by cells, such as the Free Ion activity Model (FIAM, Morel, 1983) and the Biotic Ligand Model (BLM,) are predicted on the assumption that metal internalisation rate is dependent on the concentration of the free metal in the fluid (for FIAM) or metal adsorbed on the surface (BLM). Observations generally support these assumptions but especially so when the metal concentration in solution is low (Smiejan et al., 2003). In such low metal to biomass case, the metal efflux rate is negligible with respect to the internalization rate (essentially no active regulation), which may explain the linear dependence of copper internalisation on solution concentration (Figure 2A).

However, copper is also a micronutrient that is required at low concentrations as a co-factor in multiple proteins where it participates in a range of redox reactions

251 (Tavares et al., 2006; Arguello et al., 2013), although it can also be toxic at high
252 concentrations (Rosenzweig, 2001). Its uptake and cellular concentration is therefore
253 closely regulated by a range of so-called metallochaperone proteins that collect/deliver
254 copper ions from/to specific transport P-type ATPases with high copper binding
255 affinities (Rosenzweig, 2001; González-Guerrero et al., 2008). Although cells were not
256 metabolically active (but viable) in our experiments, the observation that most copper
257 is concentrated in the periplasm at low (equilibrium) concentrations and high cell
258 concentrations is entirely consistent with an active uptake mechanism designed to
259 accumulate essential levels of copper. As relative solution concentrations increase,
260 however, the same active mechanisms will operate to reverse uptake by effluxing
261 copper to the external surface where passive biosorption (exchangeable) processes
262 operate. This switch is clearly evident in our low biomass data, (Figure 2C), and such
263 active regulation could explain the relatively low and nearly constant copper
264 concentration in the cytoplasmic space.

265 Against this argument is the observation that at the end of the adsorption
266 experiment, cells showed light blue pigmentation consistent with copper being in the
267 divalent state. Studies have shown that copper is chaperoned into/out of cells in
268 monovalent (Cu^+) state (Pufahl et al., 1997; Rosenzweig, 2001), so the blue colour
269 would contradict active regulation. It is conceivable that the blue colour represents only
270 the exchangeable component, especially as this was more evident in experiments with
271 low bacterial biomass where the exchangeable fraction was indeed high.

3.1.2 Cadmium

The behaviour of Cd at low metal to bacteria ratios is similar to that of copper in that most of the Cd resides in the EDTA-extractable fraction (periplasmic space). However, there are also subtle differences between Cd and Cu (Figure 2B). Firstly, significantly less Cd is taken up by cells compared to copper, although this could simply reflect the lower (in mole terms) total cadmium present compared to copper. Secondly, similar levels of Cd were found in exchangeable sites and in the cytoplasm at low equilibrium concentrations but the exchangeable fraction increases steeply above cytoplasmic contents at high equilibrium concentrations. The biggest difference from copper was observed for experiments using low bacteria concentrations (0.5g/L), where we measured a complete reversal in the compartmentalization of Cd between periplasmic space and exchangeable sites across all equilibrium concentrations (Figure 2D, Table 2).

As with copper, the relative partitioning of cadmium at low equilibrium concentrations is consistent with passive uptake. Unlike copper, however, cadmium is not an essential micronutrient and is extremely toxic to microorganisms, partly by interfering with cellular homeostasis of essential transition metals such as zinc (e.g. Ammendola et al., 2014). A study of Cd uptake by SmiJean et al (2003) showed that the bacterium *Rhodospirillum rubrum* responded to high Cd concentrations by actively transporting internalized Cd to the outside of the cell, as well as secreting an exudate that was able to complex Cd in solution, thereby reducing both internalized and

adsorbed Cd (Mirimanoff & Wilkinson, 2000). Our data at high solution concentrations is consistent with such active Cd regulation, except that in our case, the externalized Cd is trafficked to exchangeable sites (Figure 2D).

3.1.3 Metal efflux leads to equilibration between surface sites and cytoplasm at high surface loading

The analysis of metal partitioning from empirical equilibrium adsorption models (e.g. Langmuir) as in this study does not lend itself to evaluation of metal uptake biodynamics (internalization and efflux rates, affinity constants etc.) to enable a robust inference of the relationship between metal internalization/toxicity and the concentration of metal bound at the surface/transport sites (as required for example by the biotic ligand model). Establishing such relationships is necessary because current understanding of metal uptake dynamics assumes rapid equilibration between bulk solution and surface sites, with metal internalization being the rate-limiting process (Kola & Wilkinson, 2005); thus toxicity depends on the amount of metal bound on surface sites (Flynn & Fein, 2014). In order to provide further insight into the relationship between metal internalization and surface-site loading, we plotted internalized concentrations (q_{cyt}) against ($q_{exch} + q_{peri}$), the latter assumed to represent surface-bound metal concentration (note that plotting against q_{exch} only also gives similar relationships) that is in equilibrium with solution concentration (Figure 3).

As in Figure 3, there is a clear difference in behavior between high and low metal to biomass treatments. For high metal to biomass conditions (Figure A and B), the data

314 fits a straight line. In contrast, systems with low metal to biomass ratios are non-linear,
315 with steep slopes at low surface metal loadings that evolve to constant slopes with
316 increasing surface metal loading (Figure 3C and D).

317 A plausible interpretation of these differences/relationships is that they relate to the
318 balance between internalization and efflux rates, since these vary with metal
319 concentration/surface loading (Kola et al., 2005; Paquet et al., 2015). To illustrate this,
320 we can define the internalization rate (J_{int} , $\text{mmolkg}^{-1}\text{s}^{-1}$) with respect to this fraction as:

$$321 \quad J_{int} = k_{int}(q_{exch} + q_{per}) \quad (2)$$

322 where k_{int} is the first order internalization rate constant (s^{-1}). Similarly, the efflux rate
323 (J_{effl} , $\text{mmolkg}^{-1}\text{s}^{-1}$) can be defined with respect to the metal concentration in the
324 cytoplasm (q_{cyt}), thus:

$$325 \quad J_{effl} = k_{effl}q_{cyt} \quad (3)$$

326 where k_{effl} is the first order efflux constant (s^{-1}). Since the surface is equilibrated with
327 the solution at sampling and total metal adsorbed is time-invariant, a reasonable
328 approximation is to assume that the surface-bound metal is also in equilibrium (or at
329 least in steady-state) with the internalized metal (q_{cyt}), at least over short time scales
330 (In reality, q_{cyt} changes with time, often increasing during early exposure times
331 followed by subsequent decreases as efflux kicks in (e.g. Mairmanoff & Wlkinson,
332 2000; Smeijian et al., 2003)). Under such conditions and assuming these are single step

(elementary reactions), we can invoke microscopic reversibility (Stumm & Morgan, 1996) and equate the internalization rate to the efflux rate, thus:

$$k_{int}(q_{exch} + q_{per}) = k_{effl}q_{cyt} \quad (4)$$

such that

$$q_{cyt} = \frac{k_{int}}{k_{effl}}(q_{exch} + q_{per}) \quad (5)$$

Figure 3 is, in fact a plot of equation (5), where k_{int}/k_{effl} is the slope of the lines/curves. For high metal to biomass ratio, the straight-line relationship means that this slope can be considered as equilibrium constant (K), and partly validates our initial assumption above, since:

$$\frac{q_{cyt}}{q_{exch} + q_{per}} = \frac{k_{int}}{k_{effl}} = K \quad (6)$$

This is a new finding that needs to be checked against further experimental data but the low values of K are indicative of the dominance of metal efflux rates when cells are exposed to high metal concentrations. K values for Cu (0.053?) and Cd (0.064) are equal within the noise of the data, possibly because both metals are being actively effluxed at these high concentrations whereas because equation (5) assumes purely a passive equilibrium process. Significantly, the linear relationship also implies that metal internalization can increase beyond cellular capacity to detoxify the metal through active efflux, eventually poisoning the cell, although the observed Langmuir uptake means that q_{exch} (and q_{peri}) is constrained by the concentration of organic ligands on the surface. In contrast, it was clear in Figure 2 that Cd was severely effluxed to

exchangeable sites at these high concentrations; the most likely cause of which is active regulation. For systems with low metal to biomass ratios, internalization dominates at low surface loadings (large K), eventually switching to efflux dominance as surface loading increases (Figure 3C and D). This is entirely consistent with previous studies (e.g. Smeijian et al., 2003; Kola et al., 2005; Paquet et al., 2015).

3.1.4 Surface site ligands responsible for metal binding.

Another possible explanation for the differences between low and high metal to biomass loading may be the identity of surface active ligands present on cell surfaces that are involved in metal binding. In particular, a number of studies have shown that metal ions preferentially bind to sulfhydryl (-S-H) groups on bacterial cell surfaces at low metal to biomass ratios, with other ligands (notably carboxyl, phosphate and amine functional groups) becoming important only at high metal to biomass ratios (Mishra et al., 2010; Yu and Fein, 2015; Nell and Fein, 2017). Consequently, we determined the identity of surface ligands on *E. coli* cells by performing surface complexation modelling of potentiometric (proton adsorption) titration data, along with infrared spectroscopy.

We modelled proton binding data to 10g/L suspensions of the bacteria (data not shown) using FITEQL4 (Herbelin et al., 1999) to calculate protonation constants and surface site concentrations of surface ligands (Table 3) that can contribute to metal adsorption on cell surfaces. Consistent with previous studies (Borrok et al., 2005; Fein et al., 2005; Tourney et al., 2008; Pabst et al., 2010), the best-fitting model consisted of

four discrete proton active sites, with pK_a values of 3.48, 5.47, 7.34 and 9.97 and with cell densities of 6.25, 4.39, 1.56, 3.86×10^{-4} mol/Kg. These protonation constants are generally ascribed to phosphodiester, carboxyl, phosphate and amine/hydroxyl ligands. Sulfhydryl groups are generally thought to be too low to be detectable by this technique other through selective thiol blocking (Yu et al., 2014), but some studies have reported them using Infrared (Battol et al., 2014) and Raman (Culha et al., 2008) spectroscopy. We did not detect sulfhydryl groups on cells of *E. coli JM109* in our study, evidenced by the absence of peaks in the $2300\text{-}2500\text{ cm}^{-1}$ region (Battol et al., 2014) in Figure 4. By contrast, vibrations due to amide ($1500\text{-}1800\text{ cm}^{-1}$), carboxyl ($\sim 1400\text{ cm}^{-1}$), phosphate ($1240, 1085\text{ cm}^{-1}$), and hydroxyl ($\sim 3448\text{ cm}^{-1}$) groups are present, and their intensities decrease significantly following contact with Cu and Cd. Thus, while we can't completely rule out metal binding to sulfhydryl groups in our study, most of the metal is likely to be bound to non-sulfur ligands, particularly given that the initial metal concentrations ($> 5\text{ }\mu\text{mol/L}$) are well above those at which sulfhydryl adsorption dominates (Yu and Fein, 2015). We regard this as further evidence that metal internalisation depend on differences in metal to biomass ratio and active regulation that depends on the biotoxicity of the metal, other than differences in which ligands are involved in adsorption. Such active regulation is a common feature of viable microbial cells, including algae (e.g. Chen et al., 2010) even when cells are not actively metabolizing.

4. Conclusions

The aim of this study was to characterise metal partitioning in different cellular compartments of *E. coli* JM109 as a basis for probing the role of metal loading and the biotoxicity of the metal on metal biodynamics. Adsorption was linearly dependent on equilibrium concentrations (linear distribution co-efficient) at low metal to biomass ratio, but showed Langmuir type adsorption isotherms at high metal to biomass ratio. Distribution of each metal amongst surface sites, periplasmic space and cytoplasm depended on metal biotoxicity and metal to biomass ratio. In both cases, low metal to biomass ratio led to most of the metal being associated with the periplasmic space, with less Cd being taken up by cells overall. At high metal loading, most of the Cd was associated with surface sites, whereas Cu also increased in surface sites but remained below periplasmic concentrations. These observations are consistent with metal internalization being only dependent on the concentration of the metal in solution when metal to biomass is low and below toxic concentrations, but active efflux when metal loading is high. Significantly, efflux was more intense for high toxic element (Cd), consistent with active enzymatic regulation of Cu internalization/homeostasis, which is micronutrient element at low concentrations. Metal internalization increases as surface-bound metal increases, the maximum being constrained by maximum adsorption consistent with Langmuir adsorption behaviour. Potentiometric titrations, coupled with infrared spectroscopy the involvement of amide, carboxyl, phosphate and hydroxyl

groups in metal surface adsorption, while sulfhydryl groups were undetectable by both techniques.

Acknowledgments:

We acknowledge the hospitality accorded by the School of GeoSciences during my stay in the University of Edinburgh, and thanks Dr. Lorna Eades for help with ICP-OES analysis. This study was financially by the China Scholarship Council to Lili Liang for a one year visit to the UK(201406415064).

References

Ammendola, S., Cerasi, M., Battistoni, A., 2014. Deregulation of transition metals homeostasis is a key feature of cadmium toxicity in Salmonella. *BioMetals*, 27(4): 703-714.

434 Argüello, J.M., Raimunda,D., Padilla-Benavides, T., 2013. Mechanisms of copper
 435 homeostasis in bacteria. *Frontiers in cellular and infection microbiology*, 3(73):

436 Batool, R., Yrjala, K., Hasnain, S., 2014. Impact of environmental stress on biochemical
 437 parameters of bacteria reducing chromium. *Brazilian Journal of Microbiology*, 45
 438 (2): 573-583.

439 Borrok, D., Turner, B.F., Fein, J.B., 2005. A universal surface complexation framework
 440 for modeling proton binding onto bacterial surfaces in geologic settings. *American*
 441 *Journal of Science*, 305: 826-853.

442 Bubba, M.D., Arias, C.A., Brix, H., 2003. Phosphorus adsorption maximum of sands
 443 for use as media in subsurface flow constructed reed beds as measured by the
 444 Langmuir isotherm. *Water Research*, 37(14): 3390-3400.

445 Campbell, P.G.C., Errécalde, O., Fortin, C., Hiriart-Baer, V.P., Vigneault, B., 2002.
 446 Metal bioavailability to phytoplankton—applicability of the biotic ligand model.
 447 *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*,
 448 133(1): 189-206.

449 Chen, Z., Zhu, L., Wilkinson, K.J., 2010. Validation of the biotic ligand model in metal
 450 mixtures: bioaccumulation of lead and copper. *Environmental Science &*
 451 *Technology* 44: 3580-3586.

452 Culha, M., Adiguzel, A., Yazici, M.M., Kahraman, M., Sahin, F., Gulluce, M., 2008.
 453 Characterization of thermophilic bacteria using surface-enhanced Raman
 454 scattering. *Applied Spectroscopy*, 62 (11): 1226-1232.

455 D'Abzac, P., Bordas, F., Van Hullebusch, E., Lens, P.N.L., Guibaud, G., 2010.
 456 Extraction of extracellular polymeric substances (EPS) from anaerobic granular
 457 sludges: comparison of chemical and physical extraction protocols. *Applied*
 458 *Microbiology and Biotechnology*, 85(5): 1589-1599.

459 Duval, J.F.L. Rotureau, E., 2014. Dynamics of Metal Uptake by Charged Soft
 460 Biointerphases: Impacts of Depletion, Internalisation, Adsorption and Excretion.
 461 *Physical Chemistry Chemical Physics*, 16: 7401-7416.

462 Duval, J.F.L., Paquet, N., Lavoie, M., Fortin, C., 2015. Dynamics of Metal Partitioning
 463 at the Cell–Solution Interface: Implications for Toxicity Assessment under
 464 Growth-Inhibiting Conditions. *Environ. Science. Technol.* 49 (11): 6625–6636.

465 Duval, J.F.L., Présent, R.M., Rotureau, E., 2016. Kinetic and Thermodynamic
 466 Determinants of Trace Metal Partitioning at Biointerphases: The Role of
 467 Intracellular Speciation Dynamics. *Physical Chemistry Chemical Physics*, 18:
 468 30415-30435.

469 Duval, J.F.L., 2017. Chemodynamics of Metal Ion Complexation by Charged
 470 Nanoparticles: A Dimensionless Rationale for Soft, Core-Shell and Hard Particle
 471 Types. *Physical Chemistry Chemical Physics*, 19: 11802-11815.

472 Fein, J.B., 2017. Advanced biotic ligand models: Using surface complexation modeling
 473 to quantify metal bioavailability to bacteria in geologic systems. *Chemical*
 474 *Geology*, 464(Supplement C): 127-136.

475 Fein, J.B., Boily, J.F., Yee, N., Gorman-Lewis, D., Turner, B.F., 2005. Potentiometric
 476 titrations of *Bacillus subtilis* cells to low pH and a comparison of modeling
 477 approaches. *Geochimica et Cosmochimica Acta*, 69(5): 1123-1132.

478 Fein, J.B., Daughney, C.J., Yee, N., Davis, T.A., 1997. A chemical equilibrium model
 479 for metal adsorption onto bacterial surfaces. *Geochimica et Cosmochimica Acta*,
 480 61(16): 3319-3328.

481 Fein, J.B., Martin, A.M., Wightman, P.G., 2001. Metal adsorption onto bacterial
 482 surfaces: development of a predictive approach. *Geochimica et Cosmochimica*
 483 *Acta*, 65(23): 4267-4273.

484 Flynn, S.L., Szymanowski, J.E.S., Fein, J.B., 2014. Modeling bacterial metal toxicity
 485 using a surface complexation approach. *Chemical Geology*, 374-375(Supplement
 486 C): 110-116.

487 Fomina, M., Gadd, G.M., 2014. Biosorption: current perspectives on concept, definition
 488 and application. *Bioresource Technology*, 160(Supplement C): 3-14.

489 Gadd, G.M., 2007. Geomycology: biogeochemical transformations of rocks, minerals,
 490 metals and radionuclides by fungi, bioweathering and bioremediation.
 491 *Mycological research*, 111(1): 3-49.

492 González-Guerrero, M., Eren, E., Rawat, S., Stemmler, T.L., Argüello, J.M., 2008.
 493 Structure of the two transmembrane Cu⁺ transport sites of the Cu⁺-ATPases.
 494 Journal of Biological Chemistry, 283(44): 29753-29759.

495 Ha, J., Gelabert, A., Spormann, A.M., Brown, G.E., 2010. Role of extracellular
 496 polymeric substances in metal ion complexation by *Shewanella oneidensis*: batch
 497 uptake, thermodynamic modelling, ATR-FTIR, and EXAFS study. *Geochimica*
 498 *Cosmochimica Acta* 74 (1): 1-15.

499 Herbelin, A.L., Westall, J.C., 1999. FITEQL 4.0: a Computer Program for
 500 Determination of Chemical Equilibrium Constants from Experimental Data;
 501 Report 99-01. Department of Chemistry Oregon State University, Corvallis.

502 Kenney, J.P.L., Fein, J.B., 2011. Importance of extracellular polysaccharides on proton
 503 and Cd binding to bacterial biomass: A comparative study. *Chemical Geology*,
 504 286(3-4): 109-117.

505 Kola, H., Wilkinson, K.J., 2005. Cadmium uptake by a green alga can be predicted by
 506 equilibrium modelling. *Environmental Science & Technology* 39: 3040-3047.

507 Langley, S., Beveridge, T.J., 1999. Effect of O-Side-Chain- Lipopolysaccharide
 508 Chemistry on Metal Binding. *Applied and Environmental Microbiology*, 65(2):
 509 489-498.

510 Liu, H., Fang, H.H.P., 2002. Extraction of extracellular polymeric substances (EPS) of
 511 sludges. *Journal of Biotechnology*, 95(3): 249-256.

512 McLean, J.E., Pabst, M.W., Miller, C.D., Dimkpa, C.O., Anderson, A.J., 2013. Effect
 513 of complexing ligands on the surface adsorption, internalization, and bioresponse
 514 of copper and cadmium in a soil bacterium, *Pseudomonas putida*. *Chemosphere*,
 515 91(3): 374-382.

516 Mirimanoff, N., Wilkinson, J., 2000. Regulation of Zn Accumulation by a Freshwater
 517 Gram-Positive Bacterium (*Rhodococcus opacus*). *Environmental science &*
 518 *technology*, 34(4): 616-622.

519 Mishra, B., Moyanov, B., Bunker, B.A., Kelly, S.D., Kemmer, K. M., Fein, J. B., 2010.
 520 High- and low-affinity binding sites for Cd on the bacterial cell walls of *Bacillus*
 521 *subtilis* and *Shewanella oneidensis*. *Geochimica Cosmochimica Acta* 74 (15):
 522 4219-4233

523 Morel, F.M., 1983. Complexation: trace metals and microorganisms. *Principles of*
 524 *Aquatic Chemistry*. Wiley, New York(1983), pp 300-309.

525 Nell, R.M., Fein, J.B., 2017. Influence of sulfhydryl groups on metal binding to bacteria.
 526 *Geochimica Cosmochimica Acta* 199: 210-221.

527 Ngwenya, B.T., Sutherland, I.W., Kennedy, L., 2003. Comparison of the acid-base
 528 behaviour and metal adsorption characteristics of a gram-negative bacterium with
 529 other strains. *Applied Geochemistry*, 18(4): 527-538.

530 Ngwenya, B.T., Magennis, M., Olive, V., Mosselmans, J.F.W., Ellam, R.M., 2010.
 531 Discrete Site Surface Complexation Constants for Lanthanide Adsorption to

532 Bacteria As Determined by Experiments and Linear Free Energy Relationships.
 533 Environmental Science and Technology, 44(2): 650-656.

534 Pabst, M.W., Miller, C.D., Dimkpa, C.O., Anderson, A.J., McLean, J.E., 2010.
 535 Defining the surface adsorption and internalization of copper and cadmium in a
 536 soil bacterium, *Pseudomonas putida*. Chemosphere, 81(7): 904-910.

537 Paquet, N., Lavoie, P., Maloney, F., Duval, J.F.L., Campbell, P.G.C., Fortin., C., 2015.
 538 Cadmium Accumulation and Toxicity in the Unicellular Alga *Pseudokirchneriella*
 539 *Subcapitata*: Influence of Metal-Binding Exudates and Exposure Time.
 540 Environmental Toxicology and Chemistry, 34, (7): 1524–1532.

541 Parker, D.R., Norvell, W.A., Chaney, R., 1995. GEOCHEM-PC—a chemical
 542 speciation program for IBM and compatible personal computers. Chemical
 543 equilibrium and reaction models(chemicalequilib): 253-269.

544 Present, R.M., Rotureau, E., Billard, P., Pagnout, C., Sohm, B., Justine, F., Gley R.,
 545 Pinheiro, J.P., Duval J.F.L., 2017. Impact of Intracellular Metallothionein on
 546 Metal Biouptake and Partitioning Dynamics at Bacterial Interfaces. Physical
 547 Chemistry Chemical Physics 19: 29114-29124.

548 Pufahl, R., Singer, C., Peariso, K., Lin, S.J., Schmidt, P., Fahrni, C., 1997. Metal ion
 549 chaperone function of the soluble Cu (I) receptor Atx1. Science, 278(5339): 853-
 550 856.

- 551 Rosenzweig, A.C., 2001. Copper delivery by metallochaperone proteins. *Accounts of*
552 *Chemical Research*, 34(2): 119-128.
- 553 Rotureau, E., Billard, P., Duval, J.F.L., 2015. Evaluation of Metal Biouptake from the
554 Analysis of Bulk Metal Depletion Kinetics at Various Cell Concentrations: Theory
555 and Application. *Environmental Science & Technology* 49, (2): 990-998.
- 556 Salt, D.E., Pickering, I.J., Prince, R.C., Gleba, D., Dushenkov, S., Smith, R.D., 1997.
557 Metal Accumulation by Aquacultured Seedlings of Indian Mustard.
558 *Environmental Science and Technology*, 31(6): 1636-1644.
- 559 Smiejan, A., Wilkinson, K.J., Rossier, C., 2003. Cd Bioaccumulation by a Freshwater
560 Bacterium, *Rhodospirillum rubrum*. *Environmental science & technology*, 37(4):
561 701–706.
- 562 Stumm, W., Morgan, J.J., 1996. *Aquatic Chemistry*. John Wiley & Sons, Inc.,
563 Chichester. 1092pp.
- 564 Tavares, P., Pereira, A.S., Moura, J.J.G., Moura, I., 2006. Metalloenzymes of the
565 denitrification pathway. *Journal of Inorganic Biochemistry*, 100(12): 2087-2100.
- 566 Tourney, J., Ngwenya, B.T., Mosselmans, J.W.F., Tetley, L., Cowie, G.L., 2008. The
567 effect of extracellular polymers (EPS) on the proton adsorption characteristics of
568 the thermophile *Bacillus licheniformis* S-86. *Chemical Geology*, 247(1): 1-15.

569 Tourney, J., Ngwenya, B.T., 2009. Bacterial extracellular polymeric substances (EPS)
570 mediate CaCO₃ morphology and polymorphism. Chemical Geology, 262(3–4):
571 138-146.

572 Wu, S.C., Peng, X.L., Cheung, K.C., Liu, S.L., Wong, M.H., 2009. Adsorption kinetics
573 of Pb and Cd by two plant growth promoting rhizobacteria. Bioresource
574 Technology, 100(20): 4559-4563.

575 Yu, Q., Fein, J.B., 2015. The effect of metal loading on Cd adsorption onto *Shewanella*
576 *oneidensis* bacterial cell envelopes: The role of sulfhydryl sites. Geochimica
577 Cosmochimica Acta 167: 1-10.

578

579

580

581

582

583

584

585

586

587

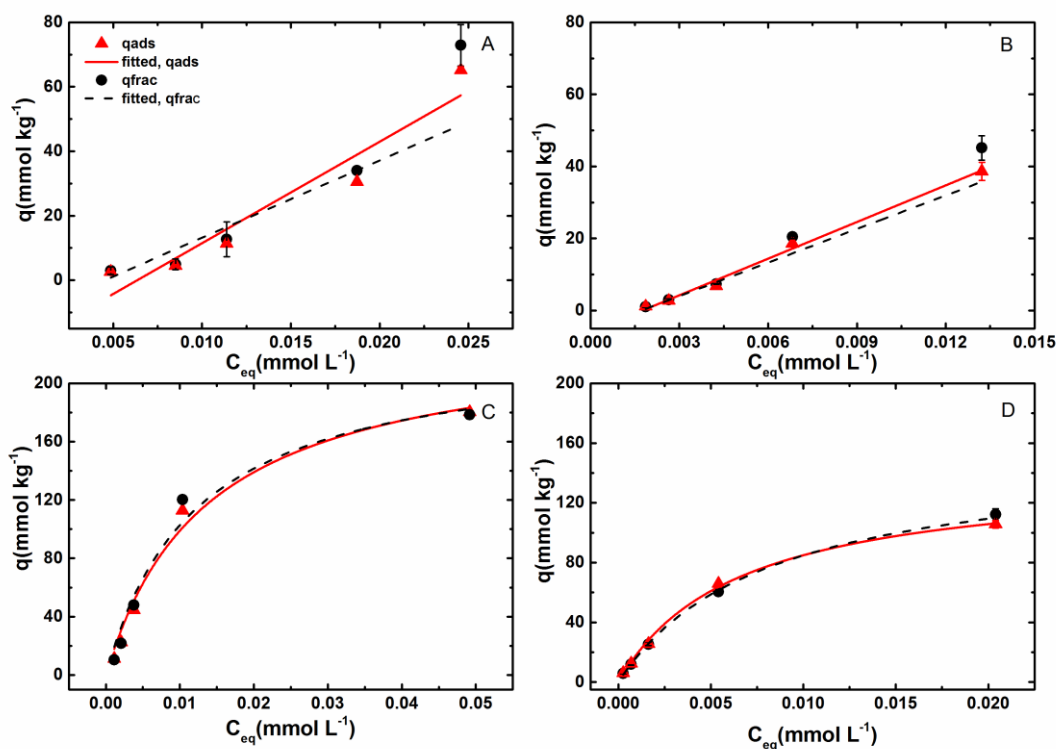


Figure 1. Sorption isotherms of Cu and Cd for low metal to biomass ratio ($2\text{g}\cdot\text{L}^{-1}$ *E.coli*-*JM109*) and high metal to biomass ratio ($0.5\text{g}\cdot\text{L}^{-1}$ *E.coli* *JM109*) suspensions, comparing amounts adsorbed from bulk solution (q_{ads}) with amounts adsorbed as calculated from mass balance of fractionated amounts (q_{frac} , the sum of q_{exch} , q_{peri} and q_{cyl}). The points are the experiment data. The lines are the Langmuir model fits. Plot A, B, C and D refer to the sorption isotherm of Cu with $2\text{g}\cdot\text{L}^{-1}$ cells, Cd with $2\text{g}\cdot\text{L}^{-1}$ cells, Cu with $0.5\text{g}\cdot\text{L}^{-1}$ cells and Cd with $0.5\text{g}\cdot\text{L}^{-1}$ cells respectively.

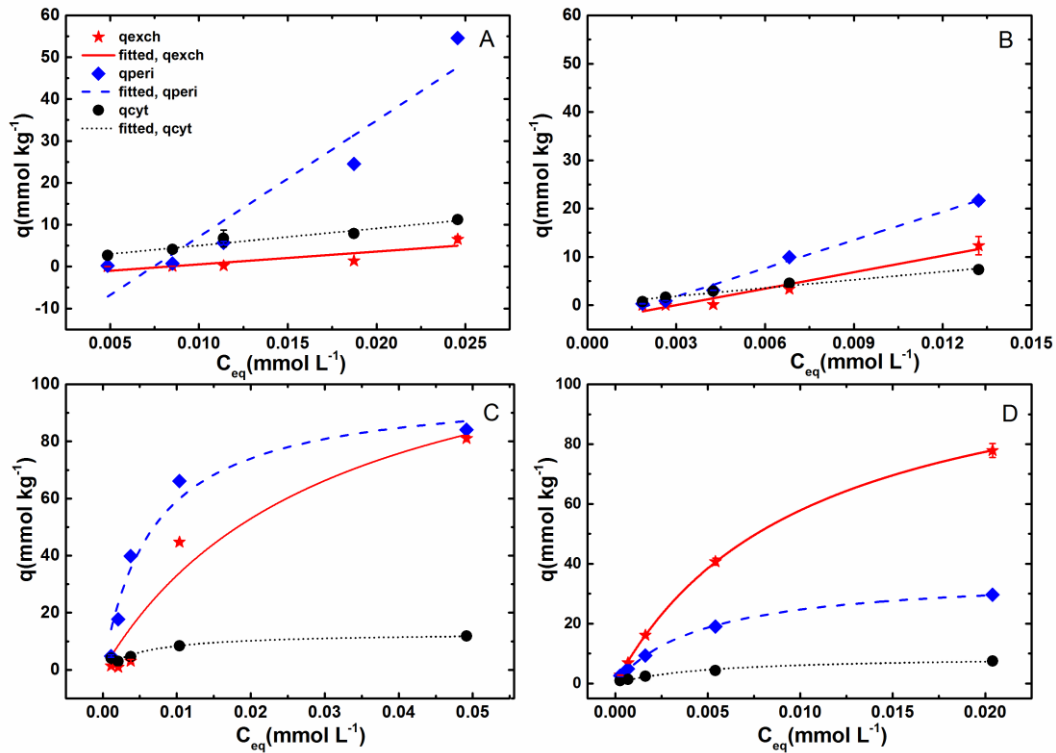


Figure 2. Sorption isotherms of Cu and Cd for low metal to biomass ratio (2g·L⁻¹ *E.coli* JM109) and high metal to biomass ratio (0.5g·L⁻¹ *E.coli* JM109) suspensions, showing amounts fractionated following bulk adsorption associated with exchangeable sites (q_{exch}), the periplasmic space (q_{peri}) and the cytoplasm (q_{cyt}) of cells. Points are the experiment data and lines are the Langmuir model fits. Plot A, B, C and D refer to the sorption isotherm of Cu with 2g·L⁻¹ cells, Cd with 2g·L⁻¹ cells, Cu with 0.5 g·L⁻¹ cells and Cd with 0.5g g·L⁻¹ cells respectively.

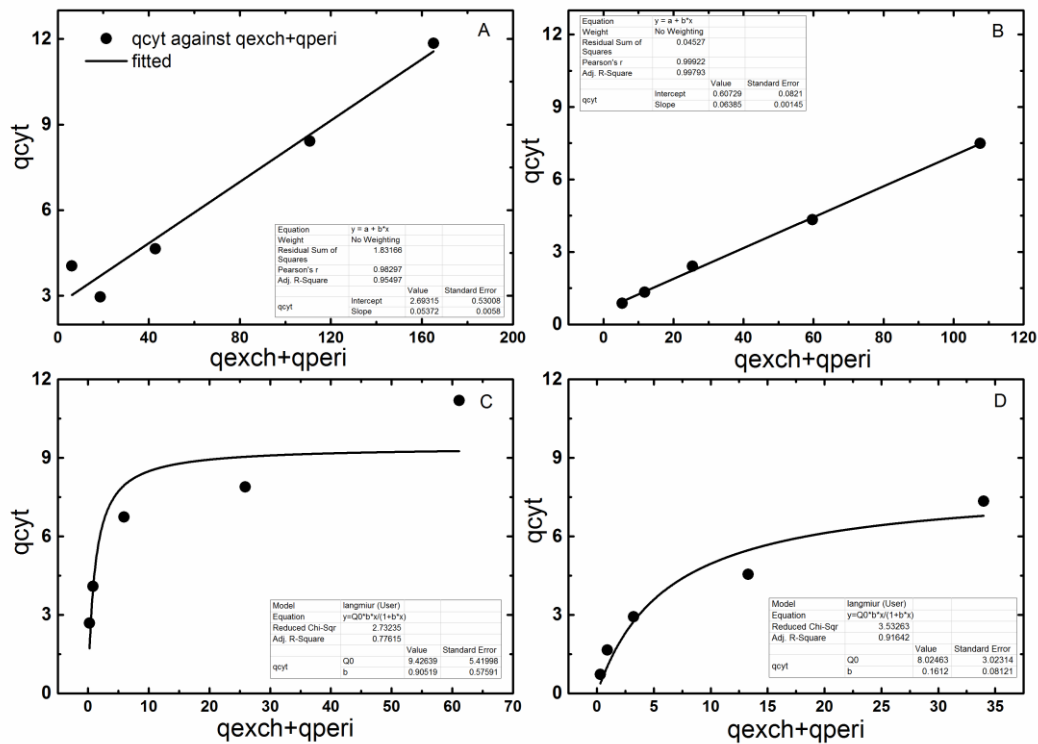


Figure 3. Graphical representation of the variation of internalised metal (q_{cyt}) against the sum of exchangeable and periplasmic concentrations ($q_{exch} + q_{peri}$) for high metal to biomass ratios (A & B, 0.5 g·L⁻¹ cells) and low metal to biomass ratio (C and D, 2 g·L⁻¹ cell) suspensions of Cu (left panels, A and C) and Cd (right panels, B and D). Suspensions with high metal to biomass ratios show a linear relationship, possibly representing a constant ratio between internalisation and efflux rates, consistent with equilibrium between internalised and surface-bound metals ($q_{exch} + q_{peri}$). Systems with low metal to biomass ratios start with high internalisation rates, which decreases as metal concentrations become toxic and efflux.

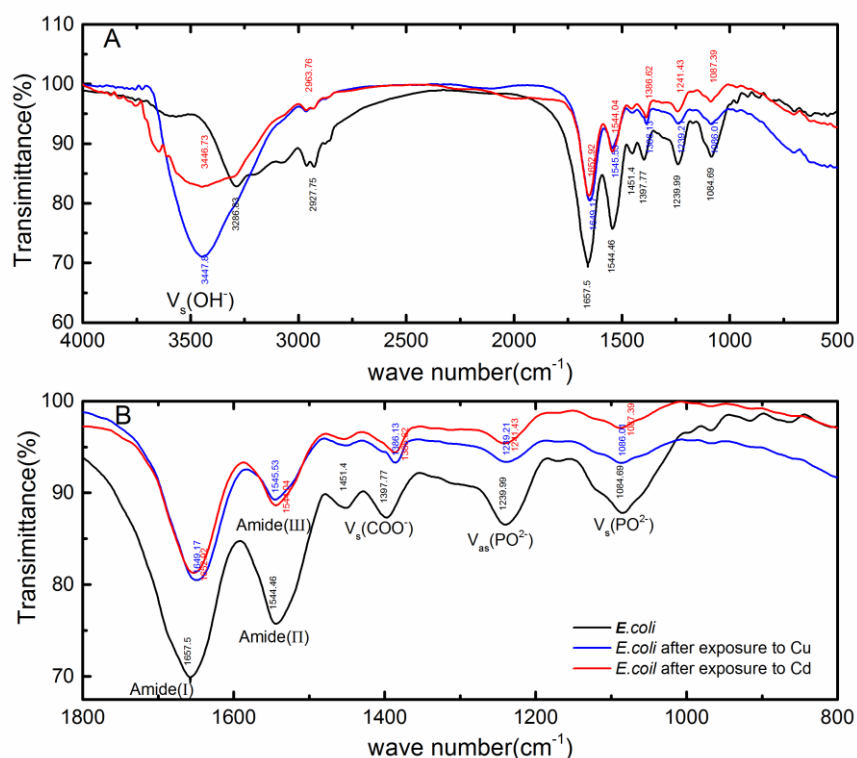


Figure 4. FTIR spectra of *E. coli* JM109 before and after exposure to Cu and Cd solutions. The concentration of Cu and Cd both are 5mg/L, background is 0.01M NaNO₃ with pH 5, and using 0.5g · L⁻¹ bacterial suspension. The black lines refer to *E. coli* without metal adsorption, blue lines and red lines refer to *E. coli* after exposure to Cu and Cd solutions respectively. Plot A is the full spectrum whereas plot B focuses on the bacterial fingerprint region.

Table 1. Langmuir parameters(Q and b) and linear parameters (Intercept and Slope), which associated with surface(qexch), periplasmic space(qperi) and cytoplasm(qcyt), for exposure 0.5g·L⁻¹ and 2 g·L⁻¹ *E.coli JM109* to Cu solution respectively.

Compartment	Q0 (mmol·kg ⁻¹)	b (L·kg ⁻¹)	Residual Sum of Squares	R ²	Percent of total Cu associated with each compartment of cell based on Q values(%)
Cu with 0.5g/L <i>E.coli-JM109</i>					
qexch [*]	134	33.0	325.8	0.916	54.3
qperi [*]	99.8	147.8	184.2	0.943	40.4
qcyt [*]	13.1	178.7	4.20	0.897	5.3
qads [#]	234	73.10	301.0	0.980	
qfrac [#]	228	82.10	506.8	0.967	
Cu with 2g/L <i>E.coli-JM109</i>					
Compartment	Intercept	Slope	R2		
qexch [*]	0.97	408.0	0.94		
qperi [*]	-20.6	2779	0.89		
qcyt [*]	-2.52	305.6	0.68		
qads [#]	-20.0	3148	0.90		
qfrac [#]	-10.8	2400	0.96		

Note: the date of * are associated with Figure 2; the date of # are associated with Figure 1.

Table 2. Langmuir parameters(Q and b) and linear parameters (Intercept and Slope), which associated with surface(qexch), periplasmic space(qperi) and cytoplasm(qcyt), for exposure 0.5g·L⁻¹ and 2 g·L⁻¹ *E.coli JM109* to Cd solution respectively.

Compartment	Q0 (mmol·kg ⁻¹)	b (L·kg ⁻¹)	Residual Sum of Squares	R ²	Percent of total Cu associated with each compartment of cell based on Q values(%)
<i>0.5g/L E.coli JM109</i> exposure to Cd					
qexch*	117	97.82	0.39	1.00	71.98
qperi*	36.4	210.7	0.66	1.00	22.38
qcyt*	9.2	197.6	0.43	0.98	5.64
qads [#]	140	153.7	12.42	1.00	
qfrac [#]	154	123.4	6.90	1.00	
<i>2g/L E.coli JM109</i> exposure to Cd					
Compartment	Intercept	Slope	R2		
qcyt*	0.21	562.0	0.96		
qperi*	-4.02	1947	0.99		
qexch*	-3.38	1136	0.94		
qads [#]	-5.93	3391	0.99		
qfrac [#]	-5.31	3106	0.92		

Note: the date of * are associated with Figure 2; the date of # are associated with Figure 1.

Table 3. Dissociation constants (pK_a where $a=1-4$) and site density (SD) in mol g^{-1} dry weight/ 10^{-4} for each site on the cells of *E. coli JM109* as determined by potentiometric titrations and FITEQL4 modelling. SD_T is the sum of the site concentrations.

Strain	pK1	SD	pk2	SD	pK3	SD	pK4	SD	SD_T	V(Y)
JM109	3.48	6.25	5.47	4.39	7.34	1.56	9.97	3.86	16.06	8.68